EFFECTS OF TEMPERATURE ON THE METABOLIC PATTERN OF INCORPORATION OF ^{14}C BY THE MOSS *DICRANUM SCOPARIUM* INCUBATED WITH ACETATE-2-^{14}C[1, 2]

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ABSTRACT

Mosses (*Dicranum scoparium*) preconditioned at both high (12°–22°C) and low (0°–10°C) ambient temperature ranges, and studied at incubation temperatures of 5°C or 22°C respectively, actively metabolized acetate-2-^{14}C. These plants malic, citric, aspartic, and glutamic acids became highly radioactive. The radioactivity was followed by solubility, column- and thin-layer chromatography, radioautography, and liquid scintillation spectrometric determinations. A change in incubation temperature brought about a qualitative change of the ^{14}C-incorporation within each metabolic pool. This qualitative change was expressed by a shift in relative radio-activity within each pool. The shifts of labeling were particularly noticeable among such individual compounds as aspartic, glutamic, malic, citric acids, glutamine, dextrose, and two lipid-fatty acid components. The data suggest that temperature affects those biochemical processes that involve the compounds which showed a shift in radioactivity. Hence, it implies that biochemical processes are closely associated with temperature changes. This study indicates that not only are Kreb cycle acids actively metabolized in mosses, but the amount found is associated with temperature changes.

INTRODUCTION

Except for the lipid-fatty acid group of compounds (James and Nichols, 1966; Canvin, 1965; and Harris and James, 1969), little is known about the metabolic pattern of non-vascular plants such as mosses under cold acclimation or stress temperatures. Many species of this group of plants are known for their hardiness and their wide distribution in the temperate as well as in the tundra habitat. Aside from being non-vascular plants, mosses have metabolic features similar to those of higher plants. They possess photosynthetic ability, and have been found to contain tricarboxylic cycle acids (TCA), amino acids, and other aromatic acids (Maass and Craigie, 1964; Walland and Kinzel, 1966; and Harris and James, 1969). An increase in unsaturation of lipids and fatty acids was observed in mosses when

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the temperature was lowered (James and Nichols, 1966; Harris and James, 1969; Wolf *et al.*, 1967; and Canvin, 1965). Moss plants with a wide range of tolerance also have greater ranges of gas exchange in photosynthesis and respiration (Rastorfer and Higginbotham, 1968). The present study examines the metabolic pattern in mosses under two different ambient temperatures: 22°C, and 5°C. The metabolic patterns were studied by the pattern of ^{14}C-incorportation into selected metabolic pools which were isolated from mosses incubated with ^{14}C-labeled substrate.

Sodium acetate-2-^{14}C was incubated with moss plants which had been preconditioned and maintained in growth chambers. Following incubation, various metabolic pools in the form of amino acids, organic acids, lipids-fatty acids, and sugars were isolated from the plant and their radioactivities determined. The evolved carbon dioxide from the incubation was trapped and each metabolic pool, which had incorporated radioactive carbon-14 was further analyzed by thin-layer chromatography (TLC) and radioautography in order to examine the ^{14}C-distribution within each metabolic pool.

MATERIALS AND METHODS

Dicranum scoparium (Hedw.), maintained in growth chambers for about 6 months at either 12° to 22° (designated "HT") or 0° to 10° (designated "LT"), were used in this study. Ten gametophytes of *D. scoparium* were placed in reaction vessels containing 7 ml of phosphate buffer solution (0.25 *M* Na_2HPO_4, pH 6.4) and kept in the growth chambers for 12 hours prior

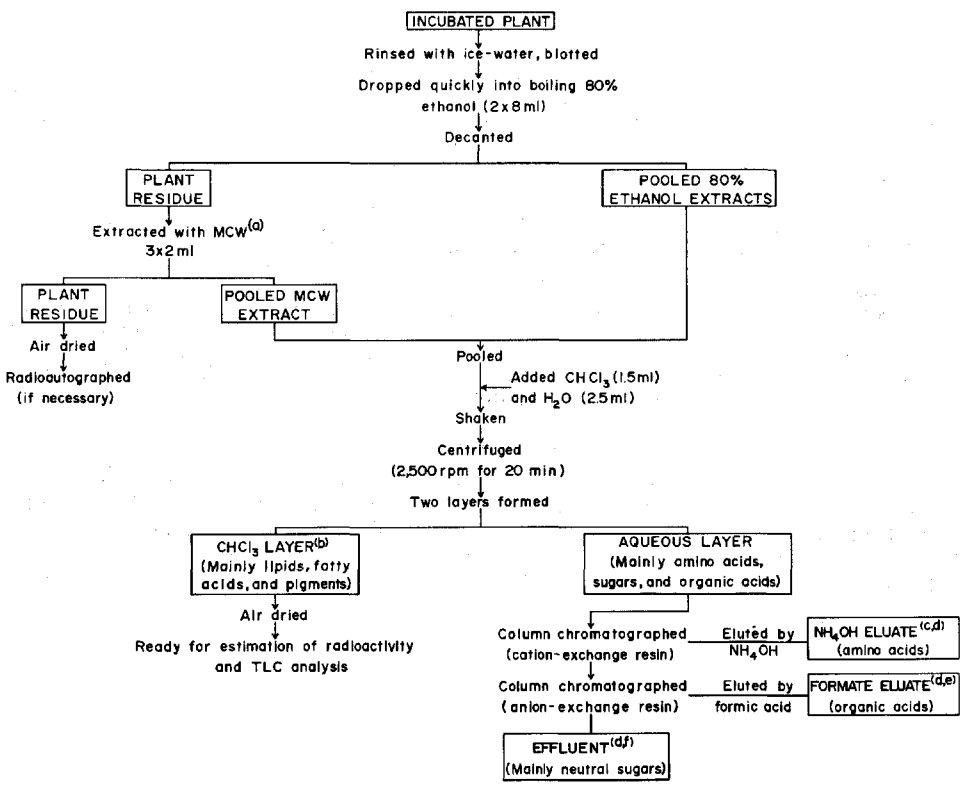


FIGURE 1. Schematic outline of the extraction and fractionation procedure used with *Dicranum scoparium* gametophytes incubated with acetate-2-^{14}C. (a) Mixture of methanol: chloroform:water (12:5:3). (b) Designated the "lipid-fatty acid pool." (c) Designated the "amino acid pool." (d) Each pool was evaporated to dryness under reduced pressure, at or below 34°C. (e) Designated the "organic acid pool." (f) Designated the "neutral sugar pool."

to the experiments. Before the gametophytes were incubated in the Gilson Differential Respirometer, 0.5 ml of 10 percent NaOH was delivered to one side-arm to trap CO_2 evolved by the plants; 0.5 ml of phosphate buffer and 200μl of sodium acetate-2-^{14}C (containing 10μc) were placed in the other side-arm. All experiments were carried out in dim laboratory lighting which was well below compensation point for the mosses. The prepared reaction vessel was incubated in the water bath of designated temperature (either 22°C or 5°C) for 120 minutes. At 60 minutes the radioactive ^{14}C-acetate (20mc/mM) was introduced by tilting the reaction vessel. Uptake of oxygen was checked at 15 minute intervals throughout the 120-minute incubation period. Experiments were carried out in duplicate.

The gametophytes were immediately removed from the vessel and the incubation mixture was subjected to the treatments outlined in figure 1, based essentially on the extraction procedure described by Bieleski and Turner (1966) and on the fractionation procedure of Shiroya *et al.* (1962). Each fraction was taken down to dryness under reduced pressure at or below 34-C. For thin layer chromatography (TLC) analysis, the organic acid-, amino acid-, and sugar-fractions were redissolved in 80%, 30–40%, or 60–70% ethanol, respectively. An aliquot of the redissolved liquid sample as well as NaOH solution was applied to a filter-paper disc, 1 cm in diameter. The disc was transferred to a counting vial containing 10 ml of scintillation fluid, which was made by mixing 100 g of naphthalene and 7 g of 2,5-diphenyloxazole (PPO) in 1 liter of 2,4-dioxane. Radioactivity was determined as counts per minute (cpm) in a Beckman liquid scintillation counter, Model CPM 100 (50% efficiency).

One dimensional TLC-analysis was done with aliquots of ^{14}C samples from the various metabolic pools, dissolved in appropriate solvents, together with authentic compounds. For the sugar pool, Eastman Chromagram 6064 Cellulose plates were used; for both the amino acid and organic acid pools, Baker-Flex Silica Gel-1B was used. The solvent-system for the organic acid pool was based on a system described by Ting and Dugger (1965). The amino acid pool was run twice in the butanol-acetic-water system of Turner and Redgwell (1966); the sugar-pool was developed in a solvent system composed of iso-propanol-n-butanol-water (7:1:2, v/v/v) (Long, 1971). The lipid-fatty acid pool was run in the system used for the organic acids on the same kind of TLC plates.

The two dimensional TLC was also used for the radioactive compounds in the amino acid pool. The procedure was essentially that of Turner and Redgwell (1966) modified to facilitate identification. Instead of having a standard amino acid mixture developed on one plate, the mixture was divided into three groups and was developed on three separate plates simultaneously, each consisting of amino acids with different Rf values in the phenol-system. In a typical experiment, two sets of TLC plates were run, and then exposed to X-ray films on which ^{14}C-spots were located. One set was used for identification by superimposing the ^{14}C-spot onto the color spot developed from the standard compound. The other set was used for radioactivity determination by scraping the ^{14}C spot off the TLC gel into a counting vial and was counted in the usual manner. Radioactivities expressed as gross activity (cpm) and relative activity (^{14}C%) were statistically evaluated by t-test and were considered at a confidence level of 95%.

It was our experience (Wu and Caldas, 1972) that pyroglutamic acid (fig. 5) also known as 2-pryolidone-5-carboxylic acid was derived from glutamic acid, when the latter was eluted from Dowex 50-ion exchange column with ammonium hydroxide. It underwent partial cyclization, therefore, the true value of glutamic acid is obtained by combining both the value of glutamic acid and its cyclized form—pyroglutamic acid.

RESULTS AND DISCUSSION

When mosses that had been preconditioned (e.g. acclimated) to high temperature at 12°–22°C (referred to as HT moss) were transferred to 5°C for incubation, there were changes in radioactivity in metabolic pools. As shown in figure 2, there was 80% decrease in respired carbon dioxide, a 33% decrease in alcoholic solubles, and a 20% increase in lipid-fatty acids. Within the alcoholic soluble fraction (fig. 3), there was a 5% increase in amino acid pool, a 5% reduction in organic acid pool, and a 50% decrease in sugar pool.

When the same mosses were preconditioned (*e.g.* acclimated) at a temperature of 0°–10°C (referred to as LT moss) and transferred to high temperature (22°C) incubation, there were changes in radioactivity of each metabolic pool examined except the lipid-fatty acid pool (fig. 2). There was an 80% increase in respired carbon dioxide, a 40% increase in alcohol soluble fractions, and no change in lipid-fatty acid fractions. Within the alcohol soluble fractions (fig. 3) there was a 50% increase in the sugar pool, a slight or no increase in the amino acid pool, and a slight or no decrease in the organic acid pool.

Temperature changes affected the ^{14}C-incorporation pattern regardless whether the mosses were preconditioned in the high temperature range (12°–22°C) or low

temperature range (0°–10°C). The degree of rise and fall in CO_2, alcohol soluble, and sugar pools of HT mosses or LT mosses were similar. Activities in the lipid-fatty acids pool and organic acid pool of LT mosses showed no change at all or only a slight change (figs. 2, 3). HT mosses had lower O_2-uptake during incubation than LT mosses (table 1). At the incubation temperature, there was a 70% reduction of O_2-uptake in HT mosses while there are only 35% increase in LT mosses. It seems that mosses acclimated in low temperature are more resistant to

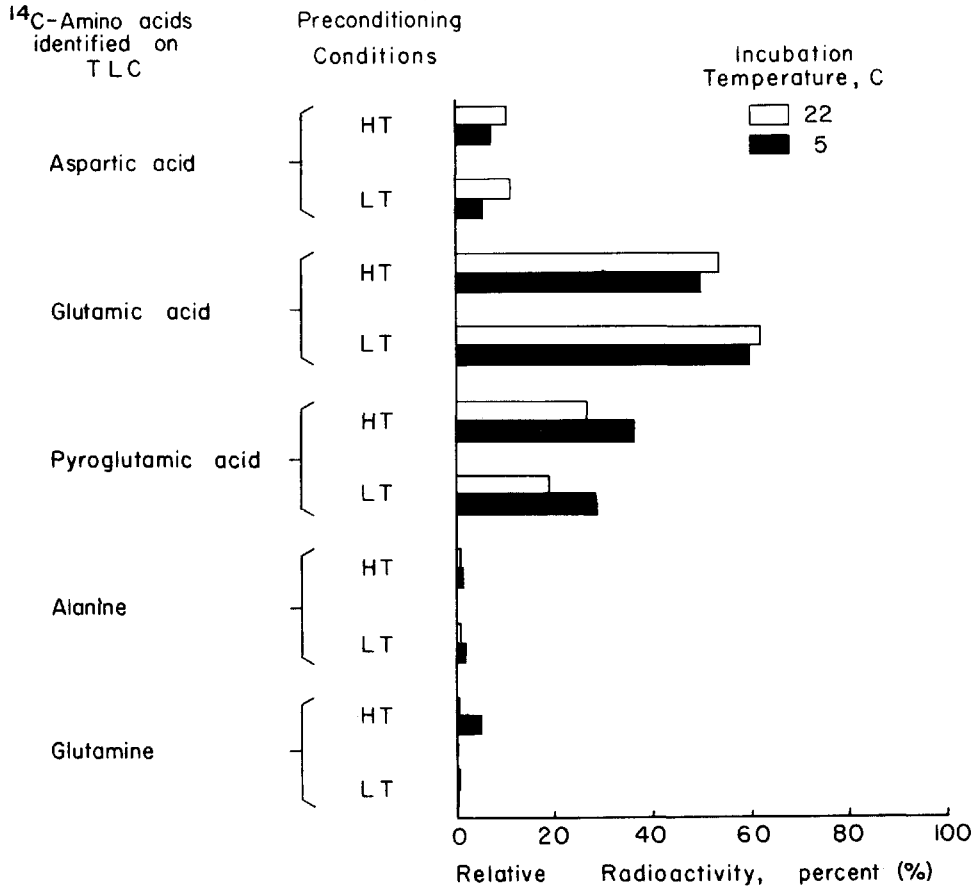
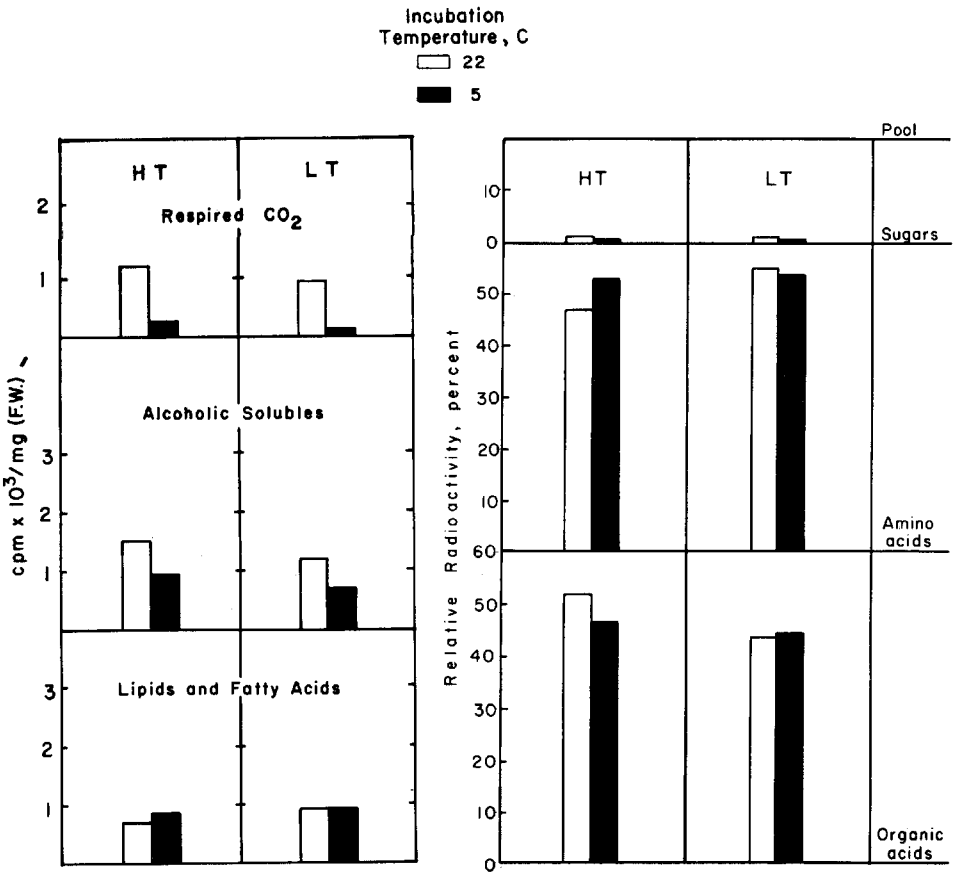


FIGURE 5. Effect of incubation temperature on the incorporation of ^{14}C from acetate-2-^{14}C into the amino acid pool of *D. scoparium* preconditioned at either high (HT) (12°C and 22°C) or low (LT) (0°C and 10°C) temperature.

environmental temperature changes. Differences were evident in labeling between mosses of the same species acclimatized (conditioned) at two different temperatures. The differences were more evident among individual ^{14}C-components. Comparisons of HT mosses at 5°C incubation with LT mosses at conditioned temperature, 5°C, showed that the activity in both glutamic acid and alanine (fig. 5) were higher while aspartic acid was lower when temperature was lowered to 5°C. This finding is in contrast to that for dogwood (Li *et al.*, 1965) in which a decrease in glutamic acid, aspartic acid, and alanine was noted at cold temperatures. The activity of glutamine remained unchanged in LT mosses but it rose sharply in HT mosses. Other individual organic acids (fig. 6) and sugars



FIGURES 2, 3. Effect of temperature on the incorporation of ^{14}C into the metabolic fractions of *D. scoparium* incubated with acetate-2-^{14}C. Incubated with acetate-2-^{14}C at either 22°C or 5°C. HT: preconditioned between 12°C and 22°C; LT: preconditioned between 0°C and 10°C. Values are for gross or relative radioactivity.

TABLE 1

Summary of experimental treatments and uptake of oxygen during each incubation

Sample	Preconditioning temperature, C[a]	Fresh weight of plants, mg	Rate of uptake of 0_2, ml/hr^{-1}g^{-1}
		Incubated at 22°C	
1	HT	187.9	148
2	HT	207.9	138
3	LT	212.6	167
4	LT	209.7	173
		Incubated at 5°C	
5	HT	187.0	20
6	HT	168.1	20
7	LT	215.3	47
8	LT	208.2	40

[a]HT indicates mosses preconditioned between 12°C and 22°C; LT indicates mosses preconditioned between 0°C and 10°C.

(fig. 7) did not show any difference. There was lower activity in the fatty component, L_1 and higher activity in component L_2 in HT mosses at 5° than those in LT mosses (fig. 4).

When HT mosses were transferred to 5° incubation there was a 40% reduction in aspartic acid (fig. 5), 60% increase in alanine (fig. 5), 25% reduction in malic acid (fig. 6), 20% increase in citric acid (fig. 6); 12% increase in gluamine (fig. 5); 40% increase in glucose (fig. 7); 15% increase, L_1, and 10% decrease, L_2 (fig. 4). When LT mosses were transferred to 22° incubation, there was a 50% increase in aspartic acid (fig. 5), 30% reduction in alanine (fig. 5), 30% increase in malic acid (fig. 6), 20% reduction in citric acid (fig. 6), no change in glutamine (fig. 5), 15% reduction in glucose (fig. 7), and 5% reduction, L_1 and 5% increase in L_2 (fig. 4). Glutamic acid and pyroglutamic acid together had the highest activity (amounting to 80%), but changed the least in both HT and LT mosses. There was only an 8% increase seen during lowering in temperature. Except for glutamine, succinic and fumaric acids, and sucrose, most of the labeling was modified in a consistent way as the temperature changed.

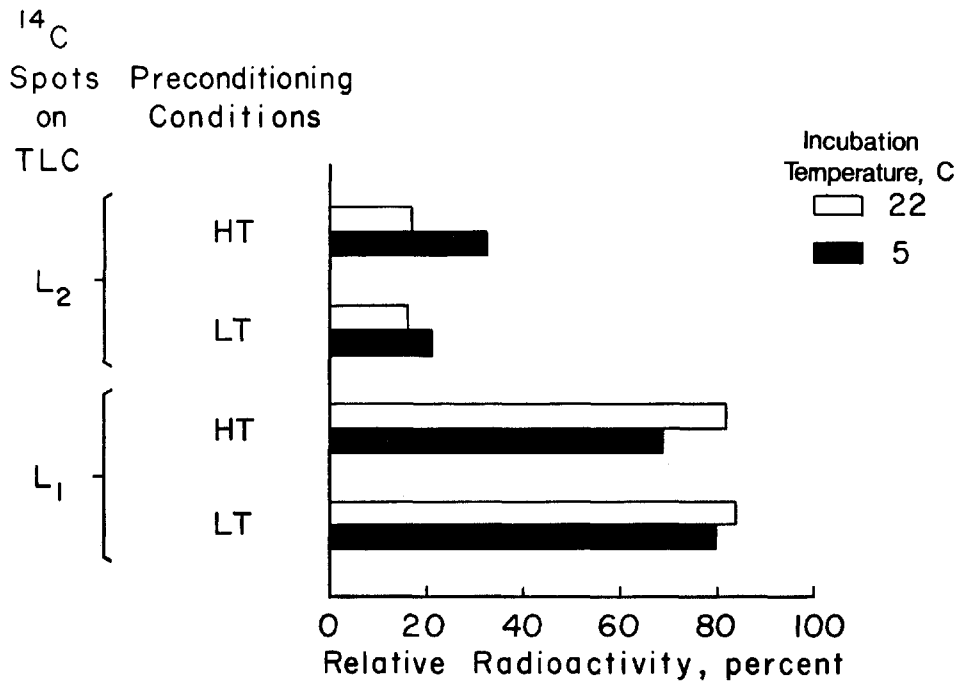


FIGURE 4. Effect of incubation temperature on the incorporation of ^{14}C from acetate-2-^{14}C into the lipid-fatty acid pool of *D. scoparium* preconditioned at either high (HT) between 12°C and 22°C or low (LT) between 0°C and 10°C temperature.

Radioactivity in the evolved carbon dioxide is an indication that the mosses under study could actively metabolize acetate-2-^{14}C just as higher plants do (Bradbeer and Colman, 1967; Harley and Beevers, 1963). The tricarboxylic cycle (TCA) acids, such as malic and citric acids and their related amino acids, aspartic, and glutamic acids, were highly radioactive (figs. 5, 6). They, together with lipid-fatty acids (fig. 4), constitute the bulk of the radioactivity in their respective pools which were from the non-protein fraction of the plants. The fact that the bulk of activity occurs in malic, citric, aspartic and glutamic acids as well as CO_2 is an indication of acetate utilization through TCA cycle (Harley and

Beevers, 1963). This suggests that non-flowering plants, such as mosses do possess metabolic process involving these acids that operate in the same way as in flowering plants, and these acids are modified by temperature changes.

The fatty component L_1 rose in high temperature, and L_2 rose in low temperature (fig. 4). Their activities show a consistent rising and lowering trend as they were modified by changes in temperature. Hence, it appears that these

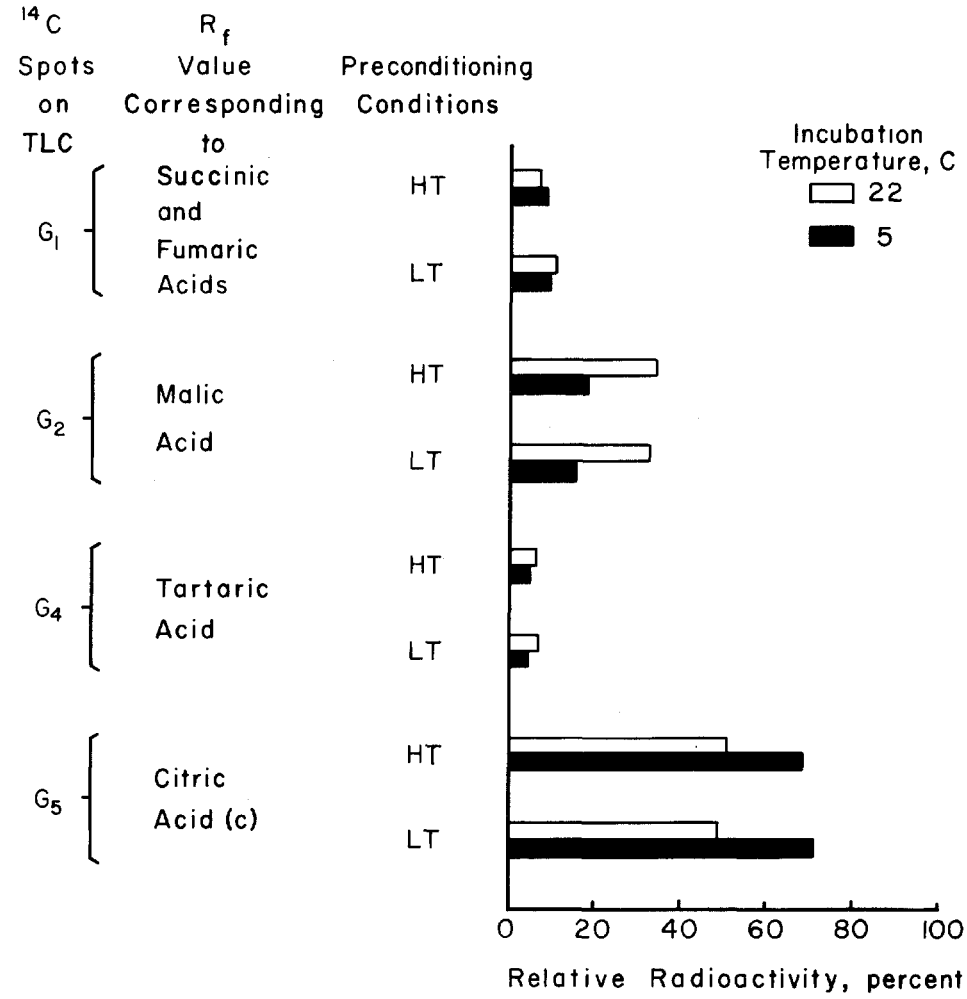


FIGURE 6. Effect of incubation temperature on the incorporation of ^{14}C from acetate-2-^{14}C into the amino acid pool of *D. scoparium* preconditioned at either high (HT) low (LT) temperature. Acids were confirmed by co-chromatography with authentic acids.

components are associated with changes of temperature, although the gross radioactivity for the whole pool revealed little difference. It would be of interest to identify these ^{14}C spots in order to make comparisons with data that have already appeared in the literature (e.g. Harris and James, 1969). The fact that there is a change in ^{14}C incorporation into lipid-fatty acids following a change of temperature, infers a change of unsaturation of fatty acids. This has been reported in mosses by others (Meyer and Block, 1963; Harris and James, 1969; and Schlenk

and Gellerman, 1965). Although as little as 2% of ^{14}C activity entered into sugar-pool from acetate-2-^{14}C in the mosses, it appears that the level of monosaccharides such as fructose and/or mannose or glucose was modified by a change of temperature, while sucrose, a disaccharide, remained unchanged in both mosses. These findings agree with those of other investigators. Glucose and sucrose have been known to be present in mosses (Mason, 1916), and sucrose showed daily increases after daylight. Similar changes were shown in higher plants, where glucose increased during cold acclimation (Li *et al.*, 1966; Parker, 1959) and sucrose remained constant (Parker, 1959).

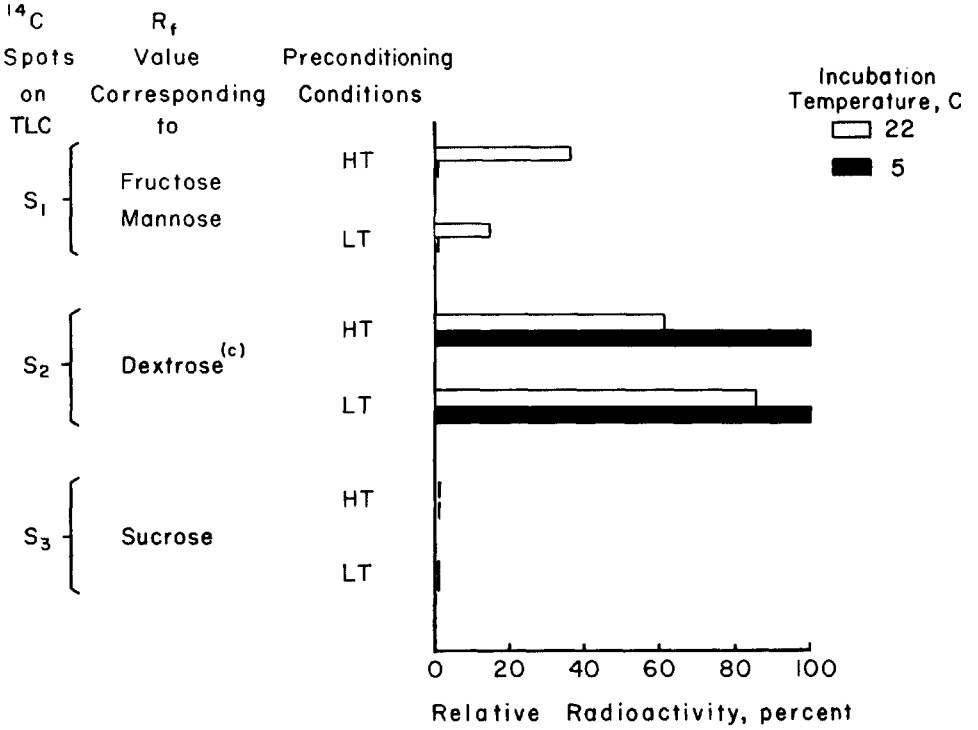


FIGURE 7. Effect of incubation temperature on the incorporation of ^{14}C from acetate-2-^{14}C into four components of the sugar pool of *D. scoparium*. Preconditioned at either high (HT) or low (LT) temperature. The identities of the sugar were confirmed by co-chromatography with authentic sugars.

Acetate-2-^{14}C was used for the first time in mosses for studying the metabolic patterns. It proved to be capable of demonstrating labeling patterns of several selected metabolic pools following changes in growing temperature. More species, especially those present in cold regions, should be tested to examine other metabolic patterns and to see how they are related to temperature variations. Future work should include the study of the protein fractions of the plant tissue. Such an approach may be helpful in probing the direct involvement of enzymes in lower plants during stress survival.

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